Effects of Short-Chain Nitrocompounds against *Campylobacter jejuni* and *Campylobacter coli* in vitro

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ABSTRACT: Effects of 2-nitro-1-propanol, 2-nitroethanol, nitroethane, and 2-nitro-methyl-propionate (0, 10, and 20 mM) on growth of *Campylobacter jejuni* were tested during culture in Bolton broth adjusted to pH 5.6, 7.0, or 8.2. The nitrocompounds were similarly tested against *C. coli* but at pH 8.2 only. Viable cell counts measured during incubation revealed main effects ($P < 0.05$) of all nitrocompounds on the survivability of *C. jejuni*. An effect of pH ($P < 0.05$) on the survivability of *C. Jejuni* during incubation with nitrocompounds was observed, with greater inhibition observed at pH 8.2 than at pH 5.6 or 7.0 for nitroethane, 2-nitro-l-propanol, and 2-nitroethanol, but not for 2-nitro-methyl-propionate, which showed greatest inhibition at pH 5.6. Except for 2-nitro-methyl-propionate, which was ineffective, all nitrocompounds elicited similar effects on *C. coli*. The effect of nitroethane and 2-nitro-l-propanol (10 mM) on naturally occurring *Campylobacter* was investigated during incubation of porcine fecal suspensions, where *Campylobacter* concentrations decreased more rapidly ($P < 0.05$) in suspensions with added 2-nitro-l-propanol than in unsupplemented or nitroethane-supplemented suspensions, thus reiterating the superior inhibitory effect of 2-nitro-l-propanol.

Keywords: *Campylobacter*; nitrocompound; preharvest food safety

Introduction

It is estimated that nearly 76 million cases of human foodborne illnesses occur in the United States each year (Mead and others 1999) at a cost of more than $7 billion annually (ERS/USDA 2001). Approximately 2.4 million of these infections have been attributed to *Campylobacter jejuni*, with 80% being foodborne transmitted (Mead and others 1999). Multiple reports have confirmed *Campylobacter* species to be the most common causes of acute bacterial diarrhea worldwide and are associated with immune-mediated neuropathies such as Guillain-Barré syndrome and Miller Fisher syndrome (Rees and others 1995; Jacobs and others 1998; Ang and others 2001).

C. jejuni accounts for approximately 99% of all campylobacter infections in the United States, leaving the other 1% to species other than *C. jejuni* (CDC 2005). Although *C. jejuni* is more commonly seen in patients with acute gastroenteritis, *Campylobacter coli,* the 2nd most prevalent species, contributed to approximately 26000 cases of intestinal inflammatory responses in 2000 (Gillespie and others 2002; Tam and others 2003). *Campylobacter* species are ubiquitous colonizers of the gastrointestinal tracts of domestic and feral animals (Jones 2001), with prevalence reported at more than 80% in swine (Pearce and others 2003) and poultry (Corry and Atabay 2001; Sahin and others 2002) and ranging from low to more than 89% in ruminants (Stanley and Jones 2003). Consequently, strategies are sought to reduce concentrations of these bacteria in animals before they arrive for processing, especially since quantitative risk assessments indicate that such interventions may significantly reduce human exposures to these pathogens (Vugia and others 2003).

Recent studies have shown that 2-nitro-1-propanol exhibits broad-spectrum antimicrobial activity against *Salmonella* serovar Typhimurium, *Escherichia coli* 0157:H7, and *Enterococcus faecalis* in vitro (Jung and others 2004a) and against *Salmonella typhimurium* when administered via oral gavage to broilers (Jung and others 2004b). Similarly, this and similar nitrocompounds have been reported to reduce gut concentrations of *Salmonella* and *Campylobacter* in pigs (Jung and others 2003), and to inhibit methane-producing activity in bovine and avian gut contents (Anderson and others 2004; Saengkerdsub and others 2006), uric acid degrading bacteria (Kim and others 2005), and *Listeria monocytogenes* in vitro (Dimitrijevic and others 2006). Reductions in animal studies have been inconsistent, however, thus suggesting that certain conditions may limit the activity of these compounds (unpublished). The present study was conducted to measure the effects of pH on the bactericidal activity of 2-nitro-1-propanol, 2-nitroethanol, nitroethane, and 2-nitro-methyl-nitroproprionate against *C. jejuni* and *C. coli* in vitro.

Materials and Methods

Bacterial strains

C. jejuni strain CC326 and *C. coli* strain CAA-39 originated from Holstein cattle (Harvey and others 2004, 2005). Isolated colonies of

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either *C. jejuni* or *C. coli* strains were incubated for 48 h on Campy-Cefex agar (Stern and others 1992), then harvested and stored in a 20% glycerol solution at −70° C when not in use. Inocula for pure test cultures were incubated overnight in Bolton Broth without antibiotics prepared with 50 mL lysed horse red blood cells/1000 mL (Lampire Biological Laboratories, Pipersville, Pa., U.S.A.).

Test conditions and incubations

Tests with pure cultures were performed using Bolton Broth adjusted to pH 5.6, 7.0, or 8.2 for the *C. jejuni* isolate and adjusted to pH 8.2 only for tests with the *C. coli* isolate via additions of 37% HCl or 5 N NaOH. 2-Nitro-1-propanol, 2-nitroethanol, nitroethane, and reagents used in the synthesis of 2-nitro-methyl-propionate were purchased from Sigma Aldrich Inc. (St. Louis, Mo., U.S.A.). 2-Nitro-methyl-propionate was synthesized by the method of Kornblum and Blackwood (1962) from methyl-bromopropionate, sodium nitrite, and phloroglucinol using dimethyl sulfoxide as the solvent. The product was distilled under vacuum from the reaction mixture as a clear liquid with a purity of 98% as determined by ^{1}H-NMR ($CDCl_3$); δ5.23 (q, 1 H, $J = 7.2$ Hz), 3.85 (s, 3 H), 1.81 (d, H, $J = 7.2$ Hz); MS, *m/e* (relative abundance) 102.0 (8), 87.1 (13), 59.0 (100), 56.0 (15), and 55.0 (13). Nitrocompounds were supplemented to 9 mL of pH adjusted Bolton broth to achieve 0, 10, or 20 mM by adding small volumes from filter sterilized (0.2 μm Acrodisc Syringe Filter, Pall Life Sciences, Ann Arbor, Mich., U.S.A.) 150 mM stock solutions prepared in distilled water. For pure culture tests, all tubes were inoculated with 10^{-2} mL of overnight grown cultures of either *C. jejuni* or *C. coli* to achieve approximately 10^6 cells/mL when brought to a final volume (10 mL) via additions of appropriately pH adjusted Bolton broth. Cultures were then incubated 48 h at 42 °C under a microaerophilic gas phase (10% CO_2, 5% O_2, and 85% N_2). The effect of 0 or 20 mM 2-nitro-1-propanol or nitroethane on naturally occurring *Campylobacter* during mixed culture was accomplished by incubating (37 °C) suspensions (10 mL) of freshly collected porcine fecal material that had been mixed 1:5 with anaerobic 0.1 *M* sodium phosphate buffer (pH 6.8) for 48 h under an anaerobic gas phase (90% N_2: 5% CO_2: 5% H_2).

Enumeration and analytical methods

Samples (1 mL) from all test incubations were collected at 0, 6, 24, and 48 h for enumeration of *Campylobacter* via plating of serial 10-fold dilutions (in 0.1 *M* phosphate buffer, pH 6.5) to Campy-Cefex agar. Colonies exhibiting typical *Campylobacter* morphology were counted after 48-h incubation. Portions of the 1:10 dilutions from the mixed culture study were also analyzed for volatile fatty acid concentrations by gas chromatography (Hinton and others 1990).

Ten representative 48-h-old colonies from the mixed culture study were randomly selected for PCR differentiation. Differentiation of these naturally occurring isolates was based on the amplification and detection of the *ceuE* gene at either 793-bp or 894-bp of *C. jejuni* or *C. coli*, respectively (Gonzalez and others 1997). Cells from each colony were added to 500 μL PCR grade H_2O in 1.5-mL microcentrifuge tubes. Samples were boiled for 10 min, then centrifuged at 8500 rpm for 15 min to isolate DNA. A master mix for amplification of each isolated colony was prepared by the addition of 25-μL Jumpstart REDTaq polymerase (Sigma-Aldrich), 1 μL of each DNA primer for *C. coli* or *C. jejuni* (Integrated DNA Technologies Inc., Coralville, Iowa, U.S.A.), and 16 μL of PCR grade H_2O (Sigma-Aldrich). Template DNA (5 μL) from each isolate was supplemented to 45 μL of the master mix to achieve a total volume of 50 μL. Electrophoresis was performed using a 2% Agarose E-gel from Invitrogen (Carlsbad, Calif., U.S.A.).

Statistical analysis

All incubations were conducted in triplicate. Effects of nitrocompound (0, 10, or 20 mM) on log transformations of *Campylobacter* concentrations, $\log_{10}$ colony forming units (CFU)/mL, during the incubations were determined by a repeated measures analysis of variance (Statistix®8 Analytical Software, Tallahassee, Fla., U.S.A.). Effects of pH on the net change of *C. jejuni* after 24-h incubation with each nitrocompound was determined by a general analysis of variance (Statistix®8 Analytical Software) with pH (5.6, 7.0, or 8.2), level of each nitrocompound (0, 10, or 20 mM) and their interaction included in the model statement. Volatile fatty acid concentrations in fluid samples collected after 24 h of the mixed culture incubations were tested for treatment effects by general analysis of variance. Means were further separated by LSD separation of means.

Results and Discussion

Numerous effective postharvest processing strategies have been employed to reduce microbial contamination of poultry and red meat carcasses (SCVPH 1998; Castell-Perez and Moreira 2004; Keeton and Eddy 2004). However, considerable interest exists for the development of preharvest strategies that can reduce the carriage of foodborne pathogens in animals prior to entering the processing plant (Callaway and others 2004). In the present study, the

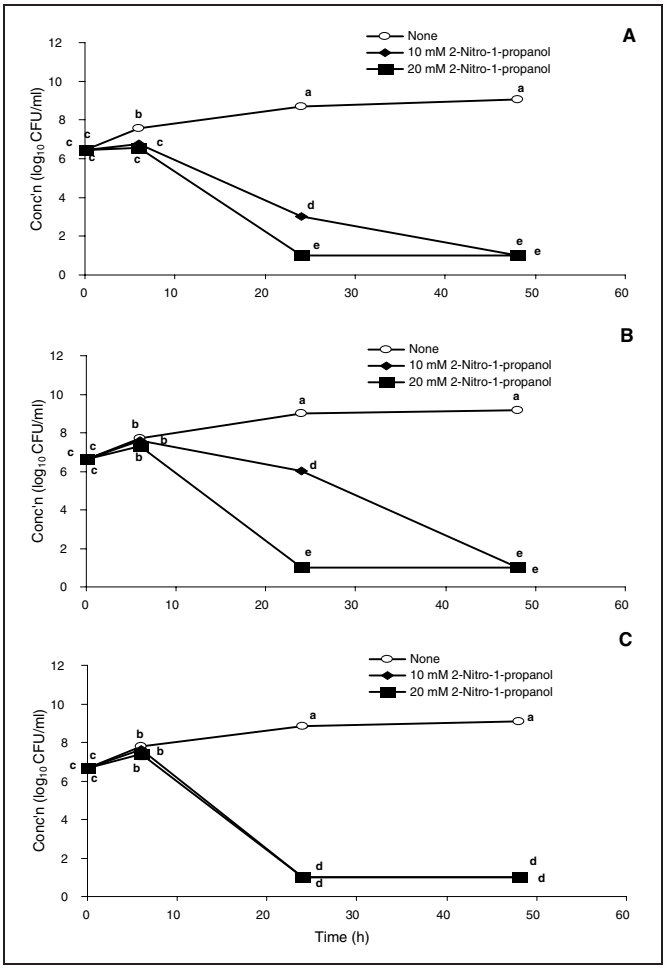

Figure 1 — Effects of 0, 10, or 20 mM 2-nitro-1-propanol on growth or survivability of *Campylobacter jejuni* during incubation in Bolton's broth adjusted to pH 5.6 (A), 7.0 (B), or 8.2 (C). Means ($n = 3$) with different letters are significantly different ($P < 0.05$); SEM = 0.10, 0.07, and 0.08 for at pH 5.6, 7.0, and 8.2, respectively.

inhibitory activity of 2-nitro-1-propanol, 2-nitroethanol, nitroethane, and 2-nitro-methyl-propionate on the survivability of *C. jejuni* during incubation in Bolton broth is evident (Figure 1 through 4), with the nitro-alcohols being more effective than the other nitroalkanes in decreasing the survivability of *C. jejuni*. The activity of the nitrocompounds, especially at the higher concentrations, appears to be bactericidal as recovery of *C. jejuni* on Campy-Cefex agar plates was markedly reduced. We cannot rule out, however, that the nitrocompounds may have induced the *Campylobacter* cells to enter into a viable but nonculturable state (Ziprin 2004) or that the selective Campy-Cefex agar may have limited the recovery of injured or stressed cells.

Effects of pH were observed on the inhibitory activity of the nitrocompounds against *C. jejuni* (Table 1). For cultures incubated with 10 mM 2-nitro-1-propanol, *C. jejuni* concentrations decreased more ($P < 0.05$) after 24-h incubation at pH 8.2 than at pH 7.0, with the net decrease of 3.45 $\log_{10}$ CFU observed for cultures incubated at pH 5.6 being intermediate ($P < 0.05$). For cultures incubated with 10 mM 2-nitroethanol, *C. jejuni* concentrations had decreased more ($P < 0.05$) after 24 h at pH 8.2 than at either pH 5.6 or 7.0. A pH effect was not observed ($P < 0.05$) in incubations with 20 mM 2-nitro-1-propanol or 2-nitroethanol. In the case of nitroethane, inhibitory activity at either 10 or 20 mM addition level was greatest ($P < 0.05$) at pH 8.2 and least ($P < 0.05$) at pH 5.6. Incubations with 20 mM 2-nitro-methyl-propionate showed greatest inhibition ($P < 0.05$) at pH 5.6 but the significant lower activity observed in incubations with 10 mM 2-nitro-methyl-propionate did not differ among the different pH conditions.

Results presented here show that 2-nitro-1-propanol and 2-nitroethanol were more effective and thus may perform better in vivo against *C. jejuni* than nitroethane or 2-nitro-methyl-propionate. These results also show that while some inhibition of *C. jejuni* was observed with all the nitrocompounds at all pH conditions tested, all except 2-nitro-methyl-propionate exhibited greatest activity at pH 8.2. The nitrocompounds possess labile protons next to the nitro group and thus may be expected to be more reactive at an alkaline pH. These findings have practical implications considering that ileal, cecal, and colonic contents of weaned pigs are typically pH 7.0 or less (Prohászka and Lukács 1984; Mathew and others 1993; Harvey and others 2001), although the pH of cecal contents in fasted pigs is more alkaline at pH 7.5 (Harvey and others 2001).

Tests of the nitrocompounds against *C. coli* yielded similar results, as inhibitory activity of 2-nitro-1-propanol, 2-nitroethanol, and nitroethane was observed, with activity being greatest ($P < 0.05$)

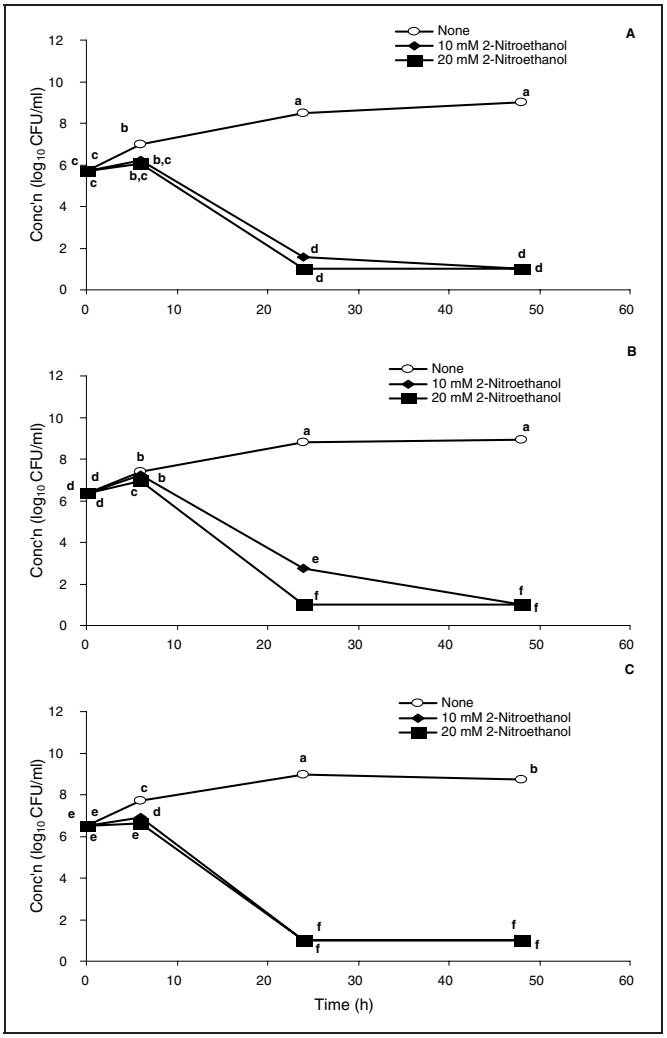

Figure 2—Effects of 0, 10, or 20 mM 2-nitroethanol on growth or survivability of *Campylobacter jejuni* during incubation in Bolton's broth adjusted to pH 5.6 (A), 7.0 (B), or 8.2 (C). Means ($n = 3$) with different letters are significantly different ($P < 0.05$); SEM = 0.35, 0.10, and 0.05 for at pH 5.6, 7.0, and 8.2, respectively.

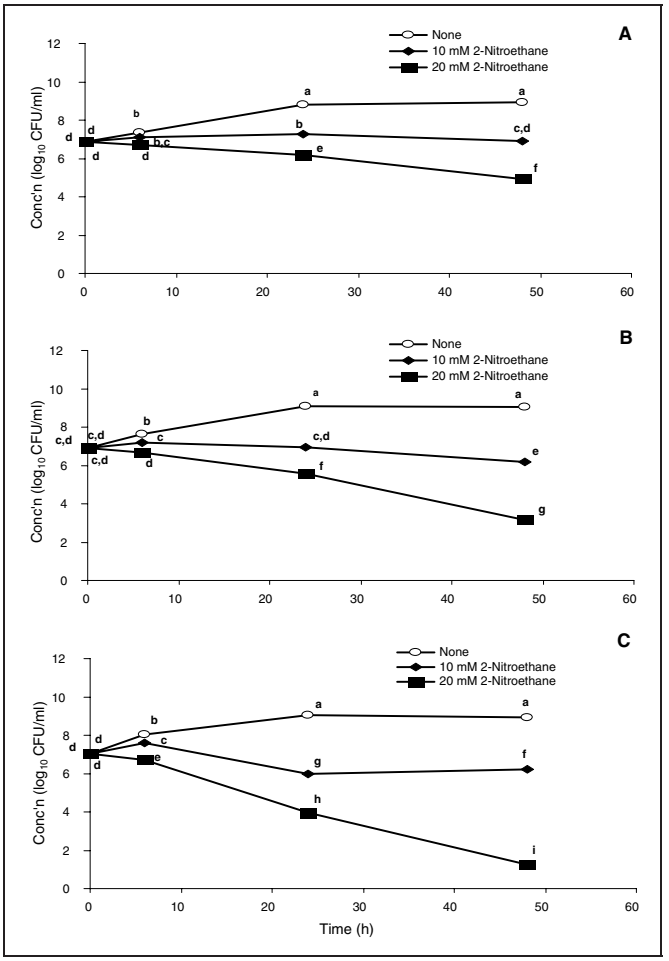

Figure 3—Effects of 0, 10, or 20 mM nitroethane on growth or survivability of *Campylobacter jejuni* during incubation in Bolton's broth adjusted to pH 5.6 (A), 7.0 (B), or 8.2 (C). Means ($n = 3$) with different letters are significantly different ($P < 0.05$); SEM = 0.08, 0.10, and 0.08 for at pH 5.6, 7.0, and 8.2, respectively.

at the higher addition level (Figure 5). Incubations containing 2-nitro-methyl-propionate showed little to no detectable activity on growth inhibition with *C. coli* species (data not shown). Based on our previous results demonstrating that a higher pH had greater inhibitory effect with all of the tested nitrocompounds except 2-nitro-methyl-propionate, we conducted our tests with *C. coli* in medium adjusted to pH 8.2 only, which may explain the absence of activity by 2-nitro-methyl-propionate. Alternatively, the inability of 2-nitro-methyl-propionate to produce inhibitory effects may be due to the insoluble nature of the compound when added to in vitro aqueous solutions.

When fresh porcine fecal suspensions were incubated 24 h anaerobically with or without 20 mM 2-nitro-1-propanol or nitroethane, naturally occurring *Campylobacter* concentrations were reduced ($P < 0.05$) 1.16 $\log_{10}$ and 3.92 $\log_{10}$ CFU units from initial microbial concentrations, respectively (Figure 6). Control values also decreased 2.83 $\log_{10}$ CFU units ($P < 0.05$) from their initial concentration after 24-h incubation, and this decrease was greater

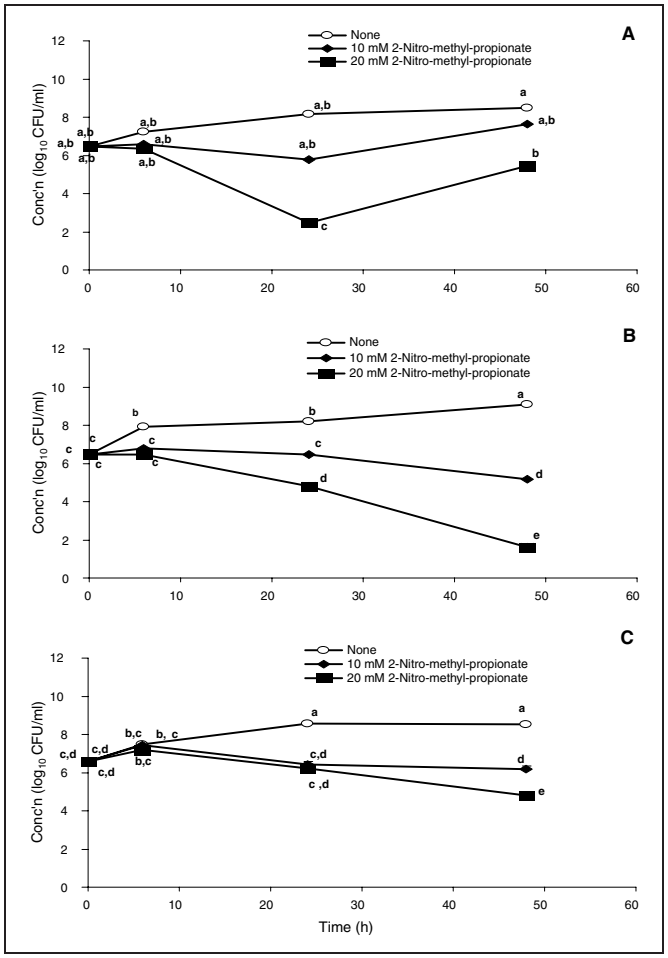

Figure 4—Effects of 0, 10, or 20 mM 2-nitro-methyl-propionate on growth or survivability of *Campylobacter jejuni* during incubation in Bolton's broth adjusted to pH 5.6 (A), 7.0 (B), or 8.2 (C). Means ($n = 3$) with different letters are significantly different ($P < 0.05$); SEM = 0.79, 0.22, and 0.27 for at pH 5.6, 7.0, and 8.2, respectively.

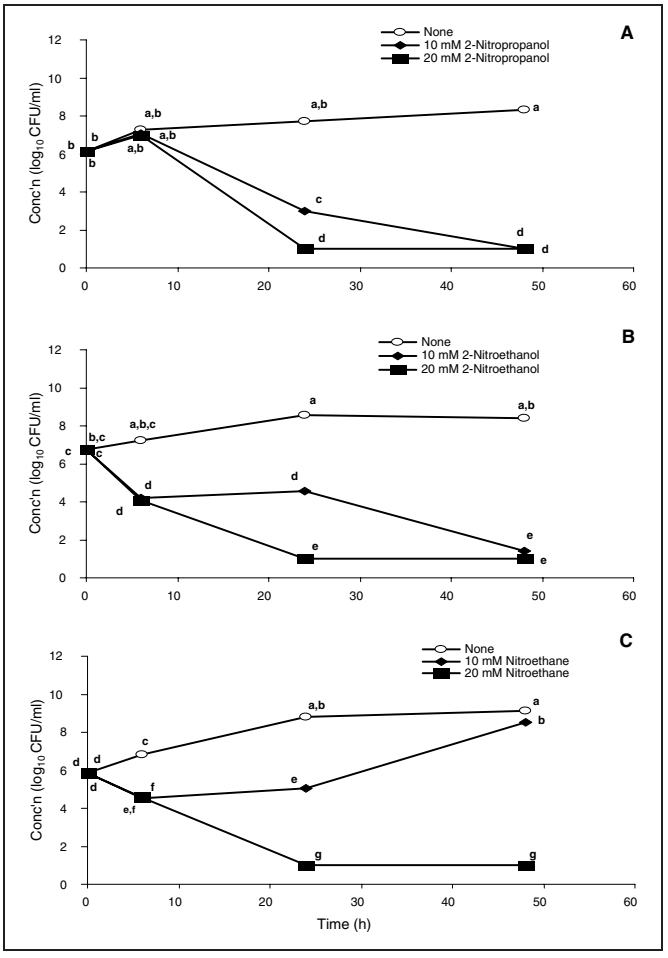

Figure 5—Effects of 0, 10, or 20 mM 2-nitro-1-propanol (A), 2-nitroethanol (B), or nitroethane (C) on growth or survivability of *Campylobacter coli* during incubation in Bolton's broth adjusted to pH 8.2. Means ($n = 3$) with different letters are significantly different ($P < 0.05$); SEM = 0.58, 0.57, and 0.15 for 2-nitro-1-propanol, 2-nitroethanol, and nitroethane, respectively.

Table 1—Main effects of nitrocompound, pH, or their interaction on net change in *C. jejuni* concentrations determined after 24-h incubation in Bolton's broth at 42 °C[a]

	2-Nitro-1-propanol (mM)			2-Nitroethanol (mM)			Nitroethane (mM)			2-Nitro-methyl-propionate (mM)		
PH	**0**	**10**	**20**	**0**	**10**	**20**	**0**	**10**	**20**	**0**	**10**	**20**
5.6	2.23^{b}	-3.45^{d}	-5.46^{e}	2.79^{f}	-4.09^{g}	$-4.68^{g,h}$	1.97^{i}	0.44^{j}	-0.68^{l}	$1.67^{o,p}$	-0.72^{q}	-4.03^{r}
7.0	2.38^{b}	-0.60^{c}	-5.64^{e}	2.46^{f}	-3.61^{g}	-5.34^{h}	2.17^{i}	0.07^{k}	-1.31^{m}	$1.73^{o,p}$	$0.10^{p,q}$	-1.65^{q}
8.2	2.18^{b}	-5.67^{e}	-5.67^{e}	2.46^{f}	-5.51^{h}	-5.51^{h}	2.03^{i}	-1.07^{m}	-3.08^{n}	1.97^{o}	$-0.14^{p,q}$	-0.36^{q}
Nitro-effect	$P < 0.0001$			$P < 0.0001$			$P < 0.0001$			$P < 0.0001$		
pH effect	$P < 0.0001$			$P = 0.03$			$P < 0.0001$			$P = 0.03$		
Interaction	$P < 0.0001$			$P = 0.14$			$P < 0.0001$			$P = 0.12$		
SEM	0.14			0.38			0.09			0.66		

[a]Tests for main effects of nitrocompound, pH, or their interaction on net change in *C. jejuni* concentrations were accomplished by general analysis of variance and a LSD separation of means. Actual concentrations of *C. jejuni* measured at time 0 and after 24-h incubation are presented in Figure 1 through 4.
[b–r]Values with different letters are significantly different ($P < 0.05$).

($P < 0.05$) than that observed in cultures containing 20 mM nitroethane. It is possible that our recovery of naturally occurring *Campylobacter* from these incubations might have been greater if we had used a microaerophilic atmosphere rather than the strict anaerobic conditions that are typically used in short-term batch cultures of mixed gut populations. Of the 10 representative colonies tested for speciation by PCR, all yielded amplicons of the *ceuE* gene indicative of *C. coli* (Gonzalez and others 1997). The observed decrease in *Campylobacter* concentrations during mixed culture incubation without added nitrocompound may be due to an accumulation of volatile fatty acids. For instance, analysis of 24-h incubation samples revealed less ($P < 0.05$) accumulation of volatile fatty acids in cultures incubated with 20 mM 2-nitro-1-propanol and nitroethane than in control cultures (Table 2). This suggested that at these concentrations the nitrocompounds may have inhibited fermentation of endogenous substrates by the anaerobic population. Decreased acetate and propionate have been associated with increased concentrations of *C. jejuni* in the swine gut (Harvey and others 2001) while increased concentrations of volatile fatty acids have been associated with decreased multiplication of *C. jejuni* in the mouse gut (Jesudason and others 1989).

Currently, aliphatic nitrocompounds such as these are used as propellants, solvents, and intermediates for organic synthesis. Secondary nitroalkanes such as 2-nitropropane and 2-nitrobutane have been shown to cause damage to rat liver DNA and RNA and to be mutagenic in their ionized form when tested by the Ames *Salmonella* assay, but primary nitroalkanes and nitrocarbinols such as 2-nitro-1-propanol were not found to be carcinogenic or mutagenic (Conaway and others 1991a, 1991b). Furthermore, toxic effects were not observed in rats following a 2-y chronic inhalation exposure to 100- or 200-ppm nitroethane (Griffin and others 1988). The oral LD_{50} of 2-nitro-1-propanol to chicks was found to be > 1300 mg/kg body weight (Jung and others 2004b). Whether the nitrocompounds can be developed for use as feed additives to control foodborne pathogens such as *Campylobacter*, *Listeria,* and *Salmonella* will undoubtedly depend on further studies examining their potential toxicity and metabolism. Precedence exists, however, for the experimental feeding of 2-nitro-1-propanol and(or) nitroethane to ruminants without any apparent adverse effects (Majak 1992; Anderson and others 2004). Additionally, earlier studies have shown that oral administration of 2-nitro-1-propanol results in significant reductions in gut *Salmonella typhimurium* and naturally occurring *Campylobacter* concentrations, thus demonstrating that this compound may have application in reducing foodborne pathogens in animals (Jung and others 2003, 2004b). In ruminants, and presumably other gut habitats, the various nitrocompounds would be expected to be reduced to their respective amines by *Denitrobacterium detoxificans,* a ruminal bacterium known to use the nitrocompounds tested here as well as 3-nitro-1-propanol and 3-nitro-1-propionic acid as terminal electron acceptors during anaerobic respiration (Anderson and others 2000).

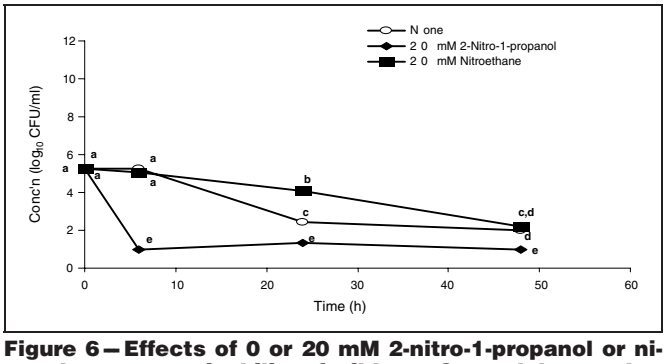

Figure 6—Effects of 0 or 20 mM 2-nitro-1-propanol or nitroethane on survivability of wildtype *Campylobacter* during anaerobic incubation of porcine fecal suspensions. Means ($n = 3$) with different letters are significantly different ($P < 0.05$); SEM = 0.14.

Table 2—Effects of nitrocompond on volatile fatty acid accumulation after 24-h incubation of freshly collected porcine feces at 37 °C

Treatment	Acetate (μmol/mL)	Propionate (μmol/mL)	Butyrate (μmol/mL)	Total (μmol/mL)
None	15.56[a]	5.27[a]	3.21[a]	24.02[a]
20 mM 2-nitro 1-propanol	9.03[b]	3.71[b]	2.07[b]	14.82[b]
20 mM nitroethane	10.24[b]	3.97[b]	2.53[b]	16.75[b]
Nitro-effect	$P = 0.02$	$P = 0.04$	$P = 0.004$	$P = 0.01$
SEM	1.15	0.34	0.14	1.47

[a,b]Values within columns with different letters are significantly different ($P < 0.05$) Tests for treatment effects were accomplished by general analysis of variance and an LSD separation of means.

Conclusion

Results presented here confirm the bactericidal activity of select nitrocompounds against *C. jejuni* and *C. coli* in vitro. For *C. jejuni*, inhibitory effects of all nitrocompounds, with the exception of 2-nitro-methyl-propionate, were greatest at pH 8.2. For *C. coli*, which was tested only at pH 8.2, the greatest inhibitory effects were seen when 20 mM of nitrocompound were added to Bolton broth. Concentrations of naturally occurring *Campylobacter,* shown by PCR analysis to be *C. coli,* decreased more rapidly during incubation of mixed fecal bacteria with 20 mM 2-nitro-1-propanol than without added nitrocompound or with 20 mM nitroethane, thus demonstrating the superior bactericidal activity of the nitro-alcohol. Although these nitrocompounds have shown significant inhibitory effects, their mechanism of action has yet to be determined. Results from this study demonstrate that growth inhibition of *C. jejuni* and *C. coli* by the nitrocompounds tested here is pH and concentration dependent. Research is under way with these and other nitrocompounds to determine whether they exhibit inhibitory activity against other foodborne pathogens and to better understand the limits of their activity.

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